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Biochemical and Molecular Analysis in a Patient With the Severe Form of Hunter Syndrome After Bone Marrow Transplantation

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Hunter syndrome (mucopolysaccharidosis type II, or MPS II) results from a deficiency of iduronate-2-sulfatase (IDS) activity due to a primary genetic defect in the X-chromosomal iduronate-2-sulfatase gene. We have studied a 10-year-old male, diagnosed with Hunter syndrome at age 2 years, who underwent bone marrow transplantation (BMT) at age 5 years. To evaluate the metabolic effect of BMT, biochemical and enzymatic studies were performed. Urinary glycosaminoglycans (GAGs) were quantitated, and iduronate-2-sulfatase activity was measured in serum, leukocytes, and liver homogenates. Decreased urinary glycosaminoglycan excretion and increased iduronate-2-sulfatase activity in serum and leukocytes were observed. Furthermore, molecular analysis was performed using reverse transcriptional polymerase chain reaction (RT-PCR) sequencing and restriction enzyme assay. The patient was found to have a novel nonsense mutation, L279X (TTA to TGA) in exon 6 of the IDS gene, inherited from his mother. A comparison of the DNA contents of cultured skin fibroblasts prior to BMT with leukocyte DNA after BMT showed coexisting host mutant and donor normal alleles in post-BMT leukocyte DNA. We postulate that the L279X mutation is a severe disease-causing mutation for Hunter syndrome.

KEY WORDS: mucopolysaccharidoses, Hunter syndrome, bone marrow transplantation, iduronate-2-sulfatase, nonsense mutation

INTRODUCTION

Hunter syndrome (mucopolysaccharidosis type II, or MPS II, MIM 309900) is an X-linked lysosomal storage disorder in which iduronate-2-sulfatase (IDS, EC 3.1.6.13) activity is deficient [Bach et al., 1973]. The deficiency of IDS activity results in faulty degradation of heparan sulfate and dermatan sulfate in various tissues, and in the increased excretion of these two polysaccharides in urine. Among patients with Hunter syndrome, there is a broad spectrum of clinical phenotypes. In the mildest form, patients are mentally normal and show only minimal symptoms of the disease. In contrast, severely affected patients present with typical clinical features including skeletal abnormalities, stiff joints, hepatosplenomegaly, cardiovascular insufficiency, and mental retardation, and they usually die before age 15 years [Neufeld and Muenzer, 1995]. The use of bone marrow transplantation (BMT) in the treatment of Hunter syndrome has been proposed based on several observations. First, in vitro metabolic correction of IDS-deficient fibroblasts was found after coincubation with non-Hunter fibroblasts [Fratantoni et al., 1968]. Second, transient biochemical and/or clinical improvement has been observed in some, but not all, patients with Hunter syndrome following infusion of normal plasma [DiFerrante et al., 1971] or fibroblast transplantation [Dean et al., 1979]. In patients with Hunter syndrome who underwent BMT, several consistent changes, such as a decrease in glycosaminoglycan (GAG) excretion, a decrease in hepatosplenomegaly, and an increase in joint mobility, have been observed. However, the long-term prognosis for longevity, cardiac complications, and neurologic outcome needs to be evaluated by systematic post-BMT monitoring [Krivit et al., 1990]. Recently, sequencing of the IDS cDNA coding region [Wilson et al., 1990] and characterization of IDS

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genomic organization [Flomen et al., 1993] have made possible the molecular analysis of specific genetic defects in the IDS gene. We report here a follow-up biochemical and enzymatic evaluation in a patient with the severe form of Hunter syndrome before and after BMT, and describe the molecular diagnosis of a novel nonsense mutation in exon 6 of the IDS gene in the patient.

MATERIALS AND METHODS

Clinical History

The proband, a 10-year-old male, began to show symptoms at age 2 years. Biochemical studies showed no detectable IDS activity in cultured skin fibroblasts. Prior to BMT treatment, the proband had coarse facial features, coarse hair and skin, mental retardation with IQ 44, mild to moderate cognitive retardation, hepatosplenomegaly, multiple bone abnormalities, and cardiac enlargement with normal function. The proband was diagnosed with a severe type of Hunter syndrome according to the clinical criteria described by Young et al. [1982], which differentiates severe from mild forms by early onset of symptoms, behavior problems, and neurological involvement. At age 5 years, the proband was treated with BMT. The donor was his human leukocyte antigens (HLA) identical brother (III-22, Fig. 1).

Urinary GAG Quantitation and IDS Activity Measurement

Specimens of urine, blood, and liver cells were obtained from the patient before and after BMT at different time intervals. Urinary GAGs were determined by a modification of the methods of Dorfman [1966]. IDS activity in serum, leukocytes, and liver cells was measured according to the method of Thompson and Nowakowski [1991].

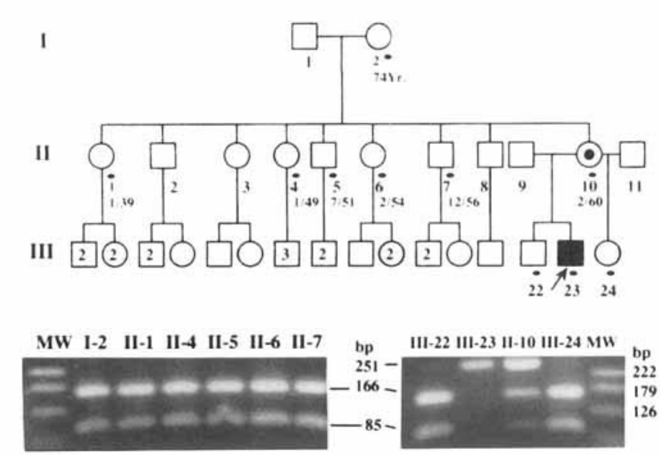

Fig. 1. Family pedigree of affected proband and carrier detection of the L279X mutation by *Mse*I digestion. Agarose gel electrophoresis shows the affected proband (III-23) (251-bp fragment not cut by *Mse*I) and his mother (II-10), who is a carrier (normal allele was cut by *Mse*I to give 166-bp and 85-bp fragments, but mutant allele remained uncut). Other relatives, i.e., I–2, II-1, II-4, II-5, II-6, II-7, III-22, and III-24, showed the normal alleles only. **Lane MW** shows the 222-bp, 179-bp, and 126-bp fragments from pGEM molecular weight markers (Promega).

RT-PCR Sequencing and Restriction Enzyme Digestion

A skin fibroblast cell line was developed from the patient for diagnosis prior to BMT. Cell lines from the patient and a normal control were used for the isolation of total RNA using RNAgents Total RNA Isolation Kit (Promega, Madison, WI). The isolated RNA was subjected to mutation analysis by a previously described RT-PCR sequencing method [Li et al., 1995]. The RT-PCR synthesized three overlapped IDS cDNA fragments flanking the entire IDS coding region and part of the 3′ nontranslated region. The three fragments were subjected to direct cycle sequencing to find mutations in the full-length IDS cDNA.

Cultured skin fibroblasts (prior to BMT) from the patient, frozen residual leukocyte pellets (3-year and 4-year post-BMT), and blood samples from several relatives of the proband were obtained for isolation of genomic DNA using the Puregene DNA isolation Kit (Gentra Systems, Inc., Minneapolis, MN). The extracted DNA served as the templates for a PCR amplification of exon 6 of the IDS gene. Two primers, 5′AAAGAGTGACAACTTTGTGGCT3′ and 5′TCATCAGTGTCCAATACATC3′, designed from the intron 5 and intron 6 sequences of the IDS gene, were used to amplify a 251-base pair (bp) fragment flanking the entire exon 6 (171 bp). PCR was performed in a 100-μl reaction mixture containing 1 μg of DNA, 50 pmole of each primer, 10 nmole each of the four nucleotides, 2 units of *Taq* polymerase (Boehringer-Mannheim Biochemicals), and buffer provided by the manufacturer. The reaction mixtures were subjected to 35 thermal cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min. PCR products were digested by restriction enzyme *Mse*I (New England BioLabs, Beverly, MA) following the manufacturer's procedure. The digested products were size-fractionated by agarose gel (1.5%) electrophoresis and visualized with ethidium bromide staining.

RESULTS

The clinical history of the proband along with the biochemical and enzymatic studies were consistent with the diagnosis of Hunter syndrome. After BMT, a decrease in enlarged liver and spleen to normal size occurred within 1 year of BMT. Three years after BMT, there were improvements in joint range of motion, and in fine and gross motor skills. However, cardiac status had not improved and mental function remained unchanged (IQ 44). Tables I and II show the results of biochemical studies in the patient. Before BMT, the patient showed a twentyfold increased urinary GAG excretion with a fivefold increase in N-sulfate content (Table I). A deficiency of IDS activity was found in serum and leukocytes (Table II). After BMT, biochemical evaluation was followed at time intervals of 1, 1.5, 3, and 4 years. Urinary GAG excretion was decreased to normal range within 1 year of BMT, and then re-

TABLE I. Measurement of Urinary GAG Before and After BMT

	Uronic acid (mg/g creatinine)[a]	N-sulfate (mg/g creatinine)[b]
Controls	14.8 ± 13.0	10.3 ± 11.1
Before BMT	323	51
1 year post-BMT	48	13
1.5 years post-BMT	47	9
3 years post-BMT	20	8
4 years post-BMT	32	4

[a]Uronic acid content was determined by the carbazole method of Dische [1947].
[b]N-sulfate content was determined by the 3-methyl-2-benzothiazione hydrazone (MBTH) method of Smith and Gilkerson [1979].

mained at normal range up to the fourth year post-BMT (Table I). IDS activity in serum showed an increase in samples from 1 and 1.5 years post-BMT, and then reached the lower range of normal IDS activity for serum in years 3 and 4 post-BMT; IDS activity in leukocytes showed a slight increase to about 8% of normal level within 1 year of BMT, and then increased to approximately 63% and 60% of normal 3 and 4 years post-BMT; liver IDS activity, measured at 1.5 years post-BMT, was about 8% of normal (Table II).

RT-PCR sequencing detected a T to G transversion at nucleotide position 836, resulting in a substitution of a stop codon (TGA) for the normal leucine (TTA) in codon 279 of IDS exon 6 (Fig. 1). The L279X mutation eliminates an *Mse*I recognition site in the mutant allele, which provides a *Mse*I digestion assay for mutation confirmation and carrier detection. Figure 3 shows the results of *Mse*I digestion on 251-bp PCR products amplified from the IDS exon 6. The 251-bp PCR fragment from the normal control was cut into 166-bp and 85-bp fragments, whereas the 251-bp fragment from the patient (skin fibroblast cell line prior to BMT) remained uncut, which confirms the L279X mutation. The 251-bp fragment from the 3-year and 4-year post-BMT leukocytes of the proband presented 251-bp, 166-bp, and 85-bp fragments upon *Mse*I digestion, which indicates the coexistence of the host mutant allele and the donor normal allele. Figure 1 shows the family pedigree of the affected proband and the results of carrier detection using the *Mse*I digestion assay. The mother (II-10) was found to be a carrier of the L279X mutation. However, neither the half-sister (III-24) nor the maternal grandmother (I-2) nor three aunts (II-1, II-4, II-6) who were studied were carriers. An older brother (III-22) and two uncles (II-5, II-7) were normal.

TABLE II. IDS Activity in Serum, Leukocytes, and Liver Before and After BMT*

	Specific activity		
	Serum	Leukocytes	Liver
Controls	20–115[a]	4–18[b]	6.3[b]
Before BMT	0.4	0	nd
1 year post-BMT	18	0.3	nd
1.5 years post-BMT	16	nd	0.5
3 years post-BMT	32	2.5	nd
4 years post-BMT	27	2.4	nd

*nd, not determined.
[a]cpm × 10^3/hr/ml.
[b]cpm × 10^3/hr/mg protein.

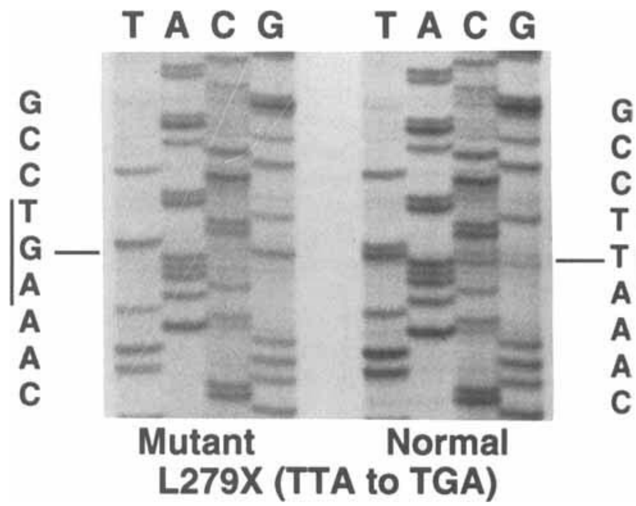

Fig. 2. Sequencing gel electrophoresis showing the L279X (TTA to TGA) mutation in exon 6 of the IDS gene.

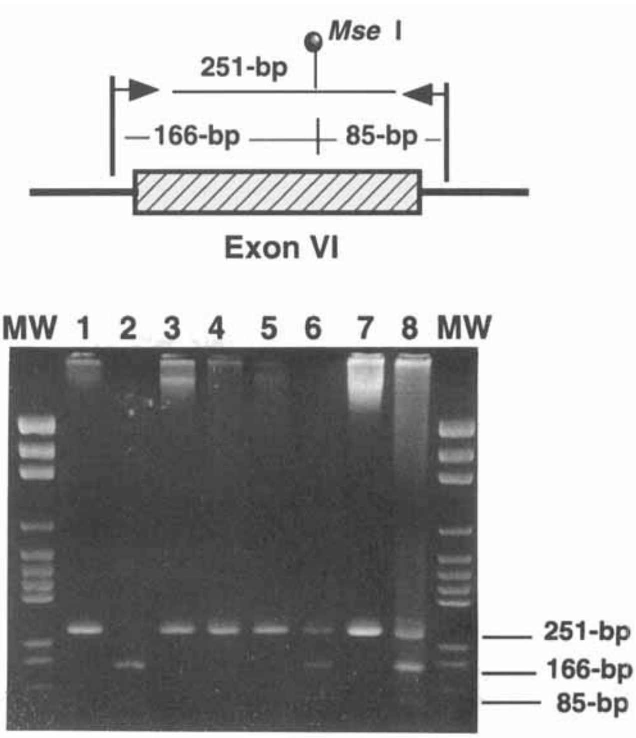

Fig. 3. Mutation analysis of proband prior to and after BMT. PCR amplification of IDS exon 6 from normal control (**lane 1**), cultured fibroblasts (**lane 3**) of the proband, and leukocyte pellets 3 years post-BMT (**lane 5**) and 4 years post-BMT (**lane 7**) of the proband showed the 251-bp products. *Mse*I digestion of these PCR products showed 166-bp and 85-bp fragments (**lane 2**), intact 251-bp (**lane 4**), and 251-bp plus 166-bp and 85-bp fragments (**lane 6** and **8**), respectively. **Lane MW**, pGEM molecular weight markers (Promega).

DISCUSSION

Biochemical studies of the proband showed decreased excretion in urinary GAGs and increased IDS activity in serum and leukocytes after BMT. A morphological observation using electron microscopy also demonstrated loss of "abnormal lysosomes" from liver hepatocytes 1.5 years post-BMT and from skin fibrocytes 3 years post-BMT [Hug et al., 1994]. The results indicate a metabolic correction produced by grafted donor cells. The metabolic correction could have occurred in two ways. First, the grafted normal cells secreted some of IDS into serum, which led to increased IDS activity in serum. The secreted enzyme could have been taken up by host leukocytes and hepatocytes, and possibly even fibrocytes through endocytosis while the enzyme was in circulation. The decrease in urinary GAG excretion occurred within 1 year post-BMT, whereas IDS activity was close to normal range for serum but only about 8% of normal activity for leukocytes. The loss of "abnormal lysosomes" in hepatocytes and fibrocytes occurred at 1.5 years and 3 years post-BMT, respectively. The decrease of urinary GAG excretion followed by the loss of "abnormal lysosomes" indicates that the secreted IDS activity in serum and the uptake of secreted IDS by hepatocytes or fibrocytes probably accounts for the corrections. Second, IDS activity in leukocytes most likely resulted from the lysosome-targeted IDS enzyme in the grafted normal cells. In contrast to the serum IDS activity, leukocyte IDS activity only achieved approximately 60% of normal 4 years post-BMT. Because the grafted normal cells were the source of secreted IDS, a relatively stable population of normal cells was required to maintain the achieved metabolic correction.

The human IDS gene is divided into nine exons and eight introns [Flomen et al., 1993]. The IDS cDNA encodes a protein of 550 amino acids and contains many areas of identical and conserved amino acids within the sulfatase enzyme group [Wilson et al., 1990]. The L279X mutation in the proband could cause a premature termination in the translational process and result in a truncated protein. The truncated protein would be predicted to be half the size of the normal IDS protein (278 amino acids out of the normal 550 amino acids). The loss of the amino terminal half of the protein would eliminate several highly conserved areas which have identical amino acid sequences in all sulfatases [Wilson et al., 1990]. We postulate that the L279X mutation is a disease-causing mutation producing a severe form of Hunter syndrome.

The L279X mutation is a novel nonsense mutation and also the first nonsense mutation reported in exon 6 of the IDS gene. Seven different nonsense mutations in the IDS gene have been reported [Bunge et al., 1992; Flomen et al., 1992; Hopwood et al., 1993; Jonsson et al., 1995]. These nonsense mutations in the IDS gene include: R172X (severe) in exon 5; W345X (severe), E375X (severe), and Q389X (severe) in exon 8; and R443X (intermediate), W475X (intermediate), and Q531X (mild), in exon 9. The nonsense mutations in the early (5′) exons of the IDS gene (one in exon 5, and our case in exon 6) are related to the severe form of the disease. Nonsense mutations in later exons were observed in patients with variable phenotypes from severe (three in exon 8), intermediate (two in exon 9), and mild (one in exon 9). A correlation between the locations of the nonsense mutations and the clinical severity could potentially be used to predict severity in Hunter syndrome. However, studies by Dietz et al. [1993] showed that nonsense mutations in the fibrillin gene and the ornithine δ-aminotransferase gene can alter in vivo splicing site selection and cause mutant exon skipping. These findings could complicate interpretation of the effect of nonsense mutations. Nevertheless, an accurate clinical documentation in patients with nonsense mutations in the IDS gene may provide some insight into the functional domains of IDS enzyme and genotype-phenotype correlation in Hunter syndrome.

Carrier testing demonstrated that the mutation was transmitted from the mother (II-10) to the proband (III-23). Other tested relatives showed only the normal allele. Also, no clinically affected patients were observed in generation, II and III from the family pedigree. Based on these results, it is reasonable to exclude the maternal grandfather (I-1) as affected. We assume that the L279X mutation originated from a germline mutation occurring in generation I (I-1, or I-2). It has been suggested that most IDS mutations are "private mutations" which occur in the affected family only [Hopwood et al., 1993]. In our patient the so-called "private mutation" probably originated from a germline mutation.

The long-term metabolic correction observed probably depends on a relatively stable population of grafted normal cells and minimal immune response to these cells. Variation in the donor/host ratio and its correlation to change in enzymatic activity could be useful in the evaluation of the post-BMT metabolic correction effect. The coexistence of the normal allele and mutant allele demonstrated by the *Mse*I digestion assay (Fig. 3) in this patient allows determination of the ratio of donor cells to host cells by a molecular approach.

REFERENCES

Bach G, Eisenberg F, Cantz M, Neufeld EF (1973): The defect in the Hunter syndrome: Deficiency of sulfoiduronate sulfatase. Proc Natl Acad Sci USA 70:2134–2138.

Bunge S, Stelich C, Beck M, Rosenkranz W, Schwinger E, Hopwood JJ, Gal A (1992): Mutation analysis of the iduronate-2-sulfatase gene in patients with mucopolysaccharidosis type II (Hunter syndrome). Hum Mol Genet 1:335–339.

Dean MF, Stevens RL, Muir H, Benson PF, Button LR, Anderson RL, Boylston A, Mowbray J (1979): Enzyme replacement therapy by fibroblast transplantation: Long-term biochemical study in three cases of Hunter's syndrome. J Clin Invest 63:138–146.

Dietz HC, Valle D, Francomano CA, Kendzior RJ, Pyeritz RE, Cutting GR (1993): The skipping of constitutive exons in vivo induced by nonsense mutations. Science 259:680–683.

DiFerrante N, Nichols BL, Donnelly PV, Neri G, Hrgovcic R, Berglund RK (1971): Induced degradation of glycosaminoglycans in Hurler's and Hunter's syndromes by plasma infusion. Proc Natl Acad Sci USA 68:303–307.

Dische Z (1947): A new specific color reaction of hexuronic acids. J Biol Chem 167:189–198.

Dorfman A (1966): The mucopolysaccharidoses. In Stanbury JB, Wyngaarden JB, Fredrickson DS (eds): "The Metabolic Basis of Inherited Disease," 2nd ed. New York: McGraw-Hill, pp 963–994.

Flomen RH, Green PM, Bentley DR, Giannelli F, Green EP (1992): Detection of point mutations and a gross deletion in six Hunter syndrome patients. Genomics 13:543–550.

Flomen RH, Green PM, Green EP, Bentley DR, Giannelli F (1993): Determination of the organisation of coding sequences within the iduronate sulphate sulphatase (IDS) gene. Hum Mol Genet 2:5–10.

Fratantoni JC, Hall CW, Neufeld EF (1968): Hurler and Hunter syndromes: Mutual correction of the defects in cultured fibroblasts. Science 162:570–572.

Hopwood JJ, Bunge S, Morris CP, Wilson PJ, Steglich C, Beck M, Schwinger E, Gal A (1993): Molecular basis of mucopolysaccharidosis type II: Mutations in the iduronate-2-sulphatase gene. Hum Mutat 2:435–442.

Hug G, Thompson JN, Chuck G, Harris R (1994): Loss of "abnormal lysosomes" in liver and skin and biochemical changes in liver, blood and urine in MPS II four years after bone marrow transplantation. Pediatr Res 35:162.

Jonsson JJ, Aronovich EL, Braun SE, Whitley CB (1995): Molecular diagnosis of mucopolysaccharidosis type II (Hunter syndrome) by automated sequencing and computer-assisted interpretation: Toward mutation mapping of the iduronate-2-sulfatase. Am J Hum Genet 56:597–607.

Krivit W, Whitley CB, Chang PN, Shapiro E, Belani KG, Snover D, Summers G, Blazar B (1990): Lysosomal storage diseases treated by bone marrow transplantation: Review of 21 patients. In Johnson FL, Pochedly C (eds): "Bone Marrow Transplantation in Children." New York: Raven Press, pp 261–287.

Li P, Huffman P, Thompson JN (1995): Mutations of the iduronate-2-sulfatase gene on a T146T background in three patients with Hunter syndrome. Hum Mutat 5:272–274.

Neufeld EF, Muenzer J (1995): The mucopolysaccharidoses. In Scriver CR, Beaudet AL, Sly WS, Valle D (eds): "The Metabolic Basis of Inherited Disease," 6th ed. New York: McGraw-Hill, pp 2465–2494.

Smith RL, Gilkerson E (1979): Quantification of glycosaminoglycan hexosamine using 3-methyl-2-benzothiazione hydrazone hydrochloride. Anal Biochem 98:478–480.

Thompson JN, Nowakowski RW (1991): Enzymatic diagnosis of selected mucopolysaccharidoses: Hunter, Morquio type A, and Sanfilippo types A, B, C, and D, and procedures for measurement of $^{35}SO_4$-glycosaminoglycans. In Hommes FA (ed): "Techniques in Diagnostic Human Biochemical Genetics: A Laboratory Manual." New York: Wiley-Liss, pp 567–586.

Wilson PJ, Morris CP, Anson DS, Occhiodoro T, Bielicki J, Clements PR, Hopwood JJ (1990): Hunter syndrome: Isolation of an iduronate-2-sulfatase cDNA clone and analysis of patient DNA. Proc Natl Acad Sci USA 87:8531–8535.

Young ID, Harper PS, Newcombe RG, Archer IM (1982): A clinical and genetic study of Hunter's syndrome. 2. Differences between the mild and severe forms. J Med Genet 19:408–411.